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MDR1 and *ERCC1* Expression Predict Outcome of Patients with Locally Advanced Bladder Cancer Receiving Adjuvant Chemotherapy[1,2,3]

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Abstract

PURPOSE: The role of adjuvant chemotherapy in patients with locally advanced bladder cancer still remains to be defined. We hypothesized that assessing the gene expression of the chemotherapy response modifiers *multidrug resistance gene 1* (*MDR1*) and *excision repair cross-complementing 1* (*ERCC1*) may help identify the group of patients benefiting from cisplatin-based adjuvant chemotherapy. *EXPERIMENTAL DESIGN:* Formalin-fixed paraffin-embedded tumor samples from 108 patients with locally advanced bladder cancer, who had been enrolled in AUO-AB 05/95, a phase 3 trial randomizing a maximum of three courses of adjuvant cisplatin and methotrexate (CM) *versus* methotrexate, vinblastine, epirubicin, and cisplatin (M-VEC), were included in the study. Tumor cells were retrieved by laser-captured microdissection and analyzed for *MDR1* and *ERCC1* expression using a quantitative real-time reverse transcription–polymerase chain reaction assay. Gene expression levels were correlated with clinical outcomes by multivariate Cox proportional hazards regression analysis. *RESULTS:* Expressions of *MDR1* and *ERCC1* were independently associated with overall progression-free survival ($P = .001$, relative risk = 2.9 and $P = .01$, relative risk = 2.24, respectively). The correlation of high *MDR1* expression with inferior outcome was stronger in patients receiving M-VEC, whereas *ERCC1* analysis performed equally in the CM and M-VEC groups. *CONCLUSIONS:* High *MDR1* and *ERCC1* gene expressions are associated with inferior outcome after cisplatin-based adjuvant chemotherapy for locally advanced bladder cancer. Prospective studies are warranted to define a role for *MDR1* and *ERCC1* analysis in individualizing multimodality treatment in locally advanced bladder cancer.

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Abbreviations: *MDR1*, *multidrug resistance gene 1*; *ERCC1*, *excision repair cross-complementing 1*; FFPE, formalin-fixed paraffin-embedded; M-VEC, methotrexate, vinblastine, epirubicin, and cisplatin; CM, methotrexate and cisplatin; Pgp, P-glycoprotein

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[2]The results of this study were presented at the American Society of Clinical Oncology Annual Meeting 2009.

[3]This is the first description of the suitability of *MDR1* and *ERCC1* expression to predict overall survival and progression-free survival in patients enrolled in a prospective randomized phase 3 trial of adjuvant chemotherapy for locally advanced bladder cancer. These findings represent an important step toward the development of biomarkers for individualizing adjuvant treatment decisions in bladder cancer patients.

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Introduction

The role of adjuvant chemotherapy in locally advanced urothelial carcinoma of the bladder is still a matter of debate. Whereas some randomized multicenter trials have demonstrated superior progression-free survival after treatment with three to four courses of methotrexate, vinblastine, adriamycin or epirubicin, and cisplatin (M-VAC/M-VEC) [1–5], no benefit in overall survival has been demonstrated. In addition, the M-VEC regimen is associated with significant toxicities, which may outweigh its potential benefits especially in elderly patients. Against this background, the AUO-AB 05/95 trial was designed to explore a deescalated adjuvant chemotherapy regimen consisting of cisplatin and methotrexate (CM). In this randomized multicenter phase 3 study, the anthracycline-containing M-VEC standard therapy failed to outperform the less toxic CM regimen [6]. However, because of the lack of an observation arm, it remains unclear whether adjuvant CM is the standard for all patients with locally advanced bladder cancer. Moreover, the superior progression-free survival after adjuvant chemotherapy in locally advanced bladder cancer could result from a strong effect in a subgroup of patients, whereas others experience no benefit. Hence, biomarkers predicting the relative risk reduction from adjuvant therapy are needed to individualize treatment strategies in bladder cancer patients.

Several gene products have been described to modify the cellular response to chemotherapeutic agents *in vitro* and to correlate with clinical outcome *in vivo*. Excision repair cross-complementing 1 (ERCC1) is a component of the nucleotide excision repair pathway, a major repair mechanism of DNA damage induced by platin compounds reacting with DNA and forming interstrand and intrastrand cross links. The balance of DNA damage to DNA repair dictates tumor cell death or survival after cisplatin therapy [7]. ERCC1 expression as detected by immunohistochemistry as well as gene expression has been linked to response and survival in many retrospective and some prospective studies in non–small cell lung cancer, colorectal cancer, and bladder cancer patients treated with platin-based therapies [8–10]. The *multidrug resistance gene 1* (*MDR1*) encodes an integral membrane protein denamed P-glycoprotein (Pgp) or an ATP-binding cassette subfamily B, member 1, which acts as an energy-dependent cellular efflux pump. Pgp was shown to reduce intracellular concentrations of a variety of cytotoxic drugs, including anthracyclines, vinca alkaloids, and taxanes. Under certain conditions, such as the presence of defective folate carrier transport proteins, methotrexate can also be a substrate of Pgp [11]. Pgp activity results in blunted chemotherapy-induced cytotoxicity *in vitro* and *in vivo*. Moreover, anticancer drugs were found to induce *MDR1* gene [12]. High Pgp levels were associated with inferior treatment outcome in elderly patients with acute myeloid leukemia [13–15], breast cancer [16,17] sarcoma [18,19], and other entities.

Thus far, only a few chemotherapy response modifiers have been assessed in small retrospective studies of bladder cancer, which failed to produce unequivocal results [20]. More than a decade ago, it was reported that M-VAC treatment of bladder cancer leads to transactivation and significantly increased expression of *MDR1*, although this result was not obtained in an outcome-driven study [21]. Analyzing tumor samples from bladder cancer patients receiving uniform adjuvant chemotherapy in a large randomized multicenter trial should increase the ability to identify truly predictive biomarkers. To this end, we retrieved formalin-fixed paraffin-embedded (FFPE) tumor samples from patients enrolled in the AUO-AB 05/95 trial [6], which compared adjuvant CM to M-VEC in 327 patients with locally advanced bladder cancer. Because both treatment arms were based on cisplatin, we focused on *ERCC1* expression. In addition, we assessed *MDR1* expression because its gene product was shown to modulate the cytotoxicity of epirubicin, vinblastine, and possibly methotrexate.

Materials and Methods

Study Population and Tumor Samples

The study population has been described previously (Table 1) [6]. Tumor staging was performed according to the criteria of the International Union Against Cancer [22].

Table 1. Patients' Demographics.

Demographic	AUO-AB 05/95 Trial Group						Study Group					
	CM (n = 163)		M-VEC (n = 164)		Total (n = 327)		CM (n = 56)		M-VEC (n = 52)		Total (n = 108)	
	n	%	n	%	n	%	n	%	n	%	n	%
Tumor category												
pTis/pT1 pN+	7	4.3	4	2.4	11	3.4	4	7.1	0	0.0	4	3.7
pT2 pN+	14	8.6	29	17.7	43	13.1	7	12.5	11	21.2	18	16.7
pT3 pN0	58	35.6	61	37.2	119	36.4	17	30.4	17	32.7	34	31.5
pT3 pN+	56	34.3	44	26.8	100	30.6	18	32.1	16	30.8	34	31.5
pT4a pN0	13	8.0	10	6.1	23	7.0	5	8.9	3	5.8	8	7.4
pT4a pN+	15	9.2	16	9.8	31	9.5	5	8.9	5	9.6	10	9.3
Nodal status												
pN0	71	43.6	71	43.3	142	43.4	22	39.3	20	38.5	42	38.9
pN+	92	56.4	93	56.7	185	56.6	34	60.7	32	61.5	66	61.1
1 lymph node	43	46.7	38	40.9	81	43.8	15	44.1	13	40.6	28	25.9
2-5 lymph nodes	37	40.2	48	51.6	85	45.9	14	41.2	17	53.1	31	28.7
>5 lymph nodes	12	13.1	7	7.5	19	10.3	5	14.7	2	6.3	7	6.5
Age, years												
≤50	26	16.0	24	14.6	50	15.3	5	8.9	11	21.2	16	14.8
51-60	61	37.4	61	37.2	122	37.3	25	44.6	19	36.5	44	40.7
61-70	76	46.6	79	48.2	155	47.4	26	46.4	22	42.3	48	44.4
Median	60.2		60.7		60.5		59.5		59		59	
Sex												
Male	123	75.5	134	81.7	257	78.6	43	76.8	42	75.0	85	78.7
Female	40	24.5	30	18.3	70	21.4	13	23.2	10	17.9	23	21.3

FFPE tissue samples were available for expression analysis from 108 of 327 study patients. The clinicopathologic characteristics of all patients were reviewed by one surgical pathologist (A.H.). Representative hematoxylin and eosin–stained slides of FFPE tissue blocks obtained at cystectomy were reviewed to estimate the tumor load per sample. For laser-captured microdissection (PALM Microlaser Technologies AG, Munich, Germany), slides of 10-μm thickness were obtained. All tumor slides were prepared as described previously [23].

Quantitative Real-time Polymerase Chain Reaction

RNA was isolated from microdissected tumor samples following a proprietary procedure at Response Genetics, Inc (Los Angeles, CA; US patent no. 6248,535). The resulting tumor RNA was reverse-transcribed into complementary DNA (cDNA) as described previously [23]. Expression of *MDR1*, *ERCC1*, and *ACTB* (β-actin, endogenous reference) was quantified by real-time fluorescence detection of amplified cDNA (ABI PRISM 7900 Sequence Detection System [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, CA). The reverse transcription–polymerase chain reaction (RT-PCR) assay was implemented as described previously [23]. All primers were selected using the Gene Express software (Applied Biosystems) but were adapted to the requirements of cDNA generated from RNA, which was extracted from the FFPE tissue. We used previously published sequences of *MDR1*, *ERCC1*, and *ACTB* [7,18,24]. All primers were validated following a previously described protocol [25]. All genes were run on all samples in triplicates, that is, one sample was run with each gene three times on the same plate to identify potential outliers. The detection of amplified cDNA results in a cycle threshold (C_t) value, which is reciprocal to the amount of cDNA contained in the sample. Normal colon, liver, and St. Universal Mix RNA (Stratagene, La Jolla, CA) were used as control calibrators on each assay plate. Gene expression levels were described as ratio between two absolute measurements (gene of interest/endogenous reference gene, here *beta-actin*) to control for intersample variation. Before statistical analysis, all ratios were logarithmically transformed including a multiplier, which accounted the average C_t values obtained for each gene during the validation process. This procedure facilitated the comparison samples, which were run on different assay plates. Depending on the used genes and mutlipliers, the interplate variation is around 5%.

Statistical Analyses

Associations of gene expression levels and progression-free or overall survival were tested for each gene by the Kaplan-Meier method. Survival differences between the high- and low-expression group were analyzed by the log-rank test. To detect independent prognostic factors associated with overall and progression-free survival, multivariate Cox proportional hazards regression analysis with stepwise selection was applied. After adjusting for potential confounders, the following parameters were accounted for: pathologic tumor stage (pT), lymph node involvement (pN), vascular invasion (V), tumor grade (G), and the gene set. In addition, receiver operating characteristic curve analysis was performed to test the ability of the chosen cut points to discriminate short survivors from long survivors [26,27].

The level of significance was set to $P < .05$. All P values reported were based on two-sided tests. All statistical analyses were performed using the Software Packages SPSS for Windows (Version 16.0; SPSS, Inc, Chicago, IL) and JMP 7.0 software (SAS, Cary, NC).

Results

Study Group and Tumor Samples

The AUO-AB 05/95 trial enrolled a total of 327 patients [6]. Tissue blocks suitable for RNA extraction were retrieved from 108 patients (33%) and subjected to further analysis. This subgroup was equally balanced for clinicopathologic parameters compared with the entire study population (Table 1). The Spearman coefficient of rank correlation of 17 staging parameters of the trial and the study group was 0.987 ($P = .0001$, 95% confidence interval [CI] = 0.964-0.995).

Gene Expression and Survival

Kaplan-Meier analysis revealed that patients with *MDR1* expression below the 75th percentile ($P = .0006$, hazard ratio [HR] = 0.25, 95% CI = 0.11-0.55) had a higher chance for prolonged survival. After 5 years, only 23% of patients with high *MDR1* expression (>75th percentile) were still alive, whereas 62% of patients with low *MDR1* expression (<75th percentile) survived 5 years. This association was still significant, when each treatment arm, CM ($P = .01$, HR = 0.26, 95% CI = 0.09-0.74; Figure 1) and M-VEC ($P = .02$, HR = 0.27, 95% CI = 0.083-0.88; Figure 2) was analyzed separately. Furthermore, patients with low *MDR1* expression had a lower risk for early progression ($P = .002$, HR = 0.28, 95% CI = 0.13-0.62). After 2 years, only 25% of patients with low *MDR1* expression experienced disease progression, whereas more than 65% of patients with high *MDR1* expression had progressed. When evaluating progression-free survival in relation to *MDR1* expression for each treatment arm, significant associations were obtained (CM: $P = .01$, HR = 0.26, 95% CI = 0.09-0.76; M-VEC: $P = .05$, HR = 0.34, 95% CI = 0.11-1.04). Next, we built a statistical model for overall survival based on *MDR1* expression, pT, pN, and pV as covariates using Cox proportional hazards regression analysis with stepwise selection (Table 2). Vascular invasion, which was apparent in 7% (8/107 patients) of the study group, was revealed as the strongest independent risk factor in this model, with a relative risk of 3.09 ($P = .02$, 95% CI = 1.19-8.03) for reduced survival time. The relative risks for high *MDR1* expression and pN2 were 2.88 ($P = .001$, 95% CI = 1.52-5.48) and 2.87 ($P = .001$, 95% CI = 1.52-5.43), respectively. Comparable results were obtained in a model for progression-free survival based on *MDR1* expression, pT, pN, and pV as covariates (Table 3).

Low *ERCC1* expression (<75th percentile) was also associated with prolonged progression-free survival ($P = .03$, HR = 0.52, 95% CI = 0.27-1.01; Figure 3). Within 5 years of follow-up, only 45% of patients with low *ERCC1* expression had progressed, whereas almost 70% of patients with high *ERCC1* expression (>75th percentile) experienced disease progression. Separate subgroup analyses of both treatment arms revealed a trend for a reduced risk of progression in patients with low *ERCC1* expression (CM: $P = .21$, HR = 0.54, 95% CI = 0.20-1.42 [Figure 4]; M-VEC: $P = .07$, HR = 0.43, 95% CI = 0.17-1.10 [Figure 5]). A significant association of *ERCC1* expression with progression-free survival (relative risk = 2.24; $P = .01$, 95% CI = 1.23-4.08) was revealed by Cox regression analysis (Table 4). Median overall survival times were 72.4 months for the low-*ERCC1* expression group and 33.1 months for the high-*ERCC1* expression group, which failed to reach significance at Kaplan-Meier analysis ($P = .19$, HR = 0.66, 95% CI = 0.35-1.24) or Cox regression analysis (relative risk = 1.75, $P = .10$, 95% CI = 0.89-3.44; Table 5). Both genes were tested together in multivariate regression models for their independent

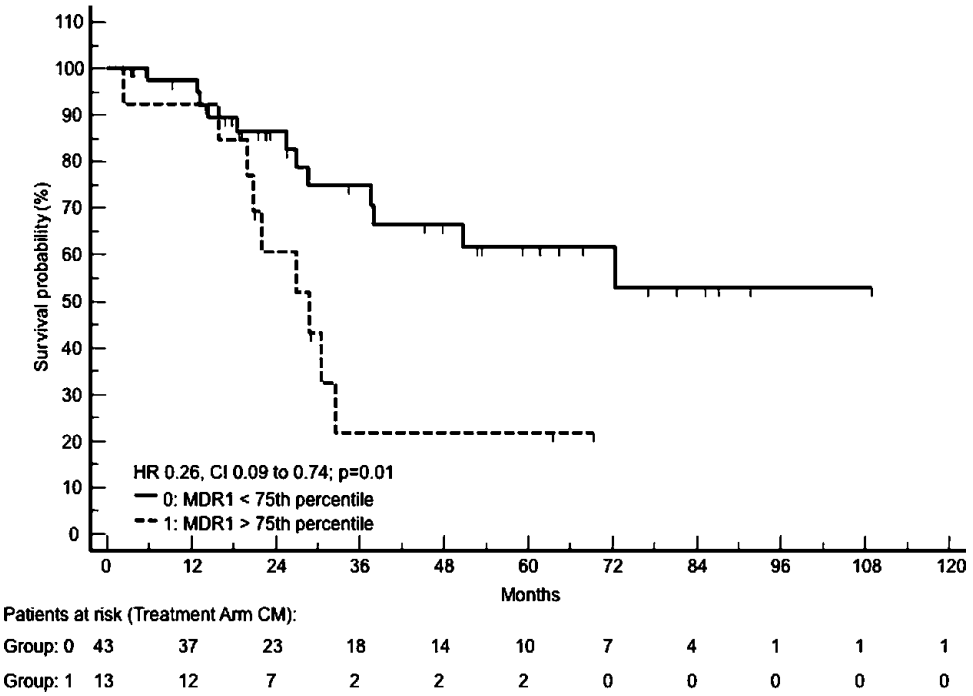

Figure 1. Kaplan-Meier plot estimating the overall survival of patients in the CM treatment arm. Differences in survival between the high- and the low-*MDR1* expression group were analyzed with the log-rank test. The upper black line represents the low-expression group, whereas the lower broken line represents the high-expression group.

association with both overall survival and progressions-free survival. However, when both genes were included in the above-mentioned model, only MDR1 remained as the most significant divisor of patients with a longer or a shorter survival (Tables 6 and 7).

Performance of MDR1 and ERCC1 Expression in Relation to Treatment Regimen

Kaplan-Meier plot analysis for overall survival revealed an early separation of the groups with high and low *MDR1* expression in patients

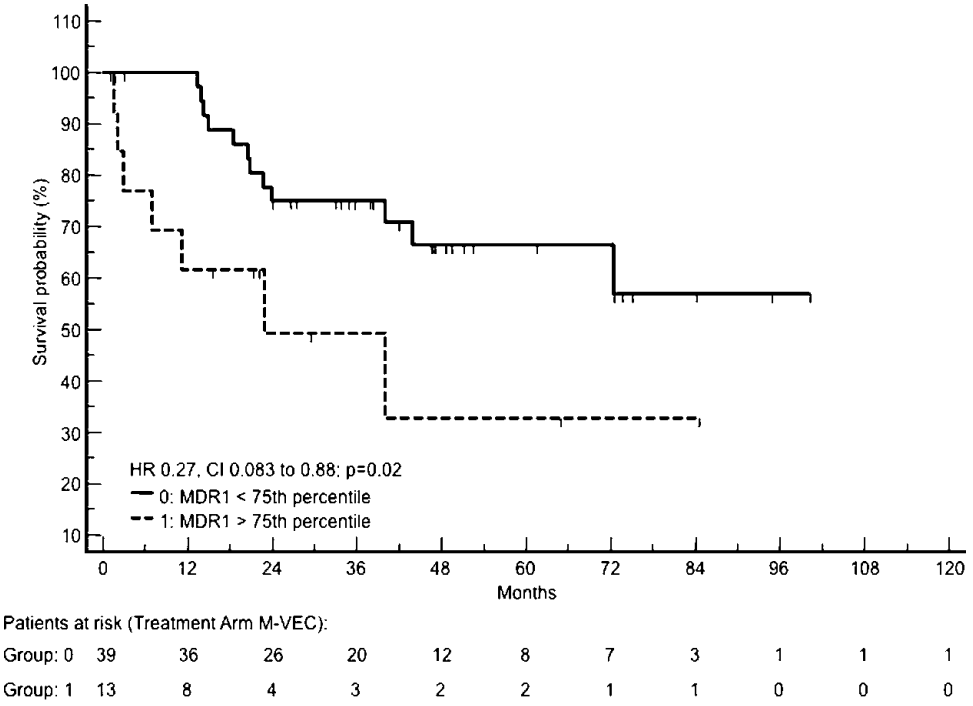

Figure 2. Kaplan-Meier plot estimating the overall survival of patients in the M-VEC treatment arm. Differences in survival between the high- and the low-*MDR1* expression group were analyzed with the log-rank test. The upper black line represents the low-expression group, whereas the lower broken line represents the high-expression group.

Table 2. Cox Proportional Hazard Regression: Overall Survival, *MDR1*.

Method	Stepwise
Enter variable if *P*	<.05
Remove variable if *P*	>.1
Sample size	107

Overall Model Fit

Null model −2 log likelihood	331.920
Full model −2 log likelihood	308.682
χ^2	23.238
df	3
Significance level	*P* < .0001

Coefficients and SE

Covariate	*b*	SE	*P*	Exp(*b*)	95% CI of Exp(*b*)
MDR1 > 75%	1.0588	0.3294	.001306	2.8829	1.5167-5.4797
pN = 2	1.0541	0.3267	.001254	2.8694	1.5174-5.4261
Vascular invasion	1.1277	0.4900	.02137	3.0886	1.1879-8.0306
Variables not included in the model					
pT = 1					
pT = 2					
pT = 4					
pN = 1					

Table 3. Cox Proportional Hazard Regression: Progression-Free Survival, *MDR1*.

Method	Stepwise
Enter variable if *P*	<.05
Remove variable if *P*	>.1
Sample size	105

Overall Model Fit

Null model −2 log likelihood	376.539
Full model −2 log likelihood	349.441
χ^2	27.097
df	3
Significance level	*P* < .0001

Coefficients and SE

Covariate	*b*	SE	*P*	Exp(*b*)	95% CI of Exp(*b*)
MDR1 > 75%	1.0478	0.3234	.001194	2.8514	1.5178-5.3567
pN = 2	1.0742	0.3071	.0004684	2.9277	1.6087-5.3283
Vascular invasion	1.3289	0.4580	.003715	3.7769	1.5461-9.2264
Variables not included in the model					
pT = 1					
pT = 2					
pT = 4					
pN = 1					

treated with M-VEC compared with CM treatment (Figures 1 and 2). In contrast, no such difference was observed when separating for *ERCC1* expression (Figures 4 and 5). Receiver operating characteristic curve analysis was applied to test whether there was a difference in the sensitivity and specificity of *MDR1* expression for discrimination of short-term survivors from long-term survivors. In the M-VEC–treated patient group, high *MDR1* expression exhibited significant (*P* = .008, area under the curve = 0.71, 95% CI = 0.56-0.82) sensitivity of 69% (true-positive rate) and specificity of 72% (true-negative rate) for discrimination between patients surviving longer than 24 months and those who died earlier. This level of significance was not observed in the CM-treated patient group (*P* = .91, area under the curve = 0.5, 95% CI = 0.37-0.65), which revealed a sensitivity of only 46% and a specificity of 56%.

Discussion

Patients experiencing bladder cancer growing beyond the lamina muscularis propria [28] and/or metastasizing to the lymph nodes have a

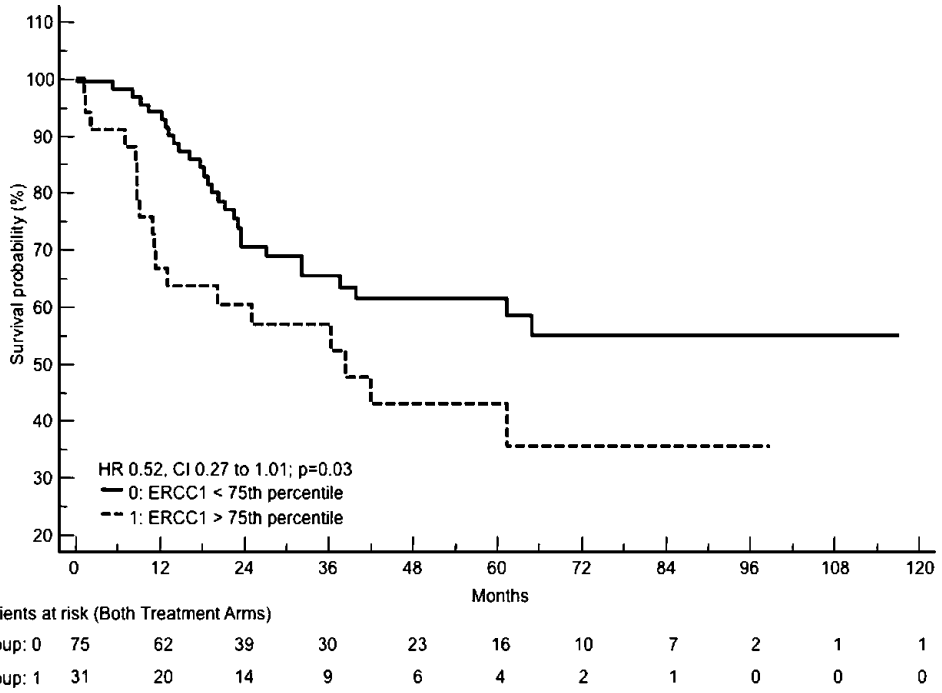

Figure 3. Kaplan-Meier plot estimating the progression-free survival of patients in both treatment arms. Differences in survival between the high- and the low-*ERCC1* expression group were analyzed with the log-rank test. The upper black line represents the low-expression group, whereas the lower broken line represents the high-expression group.

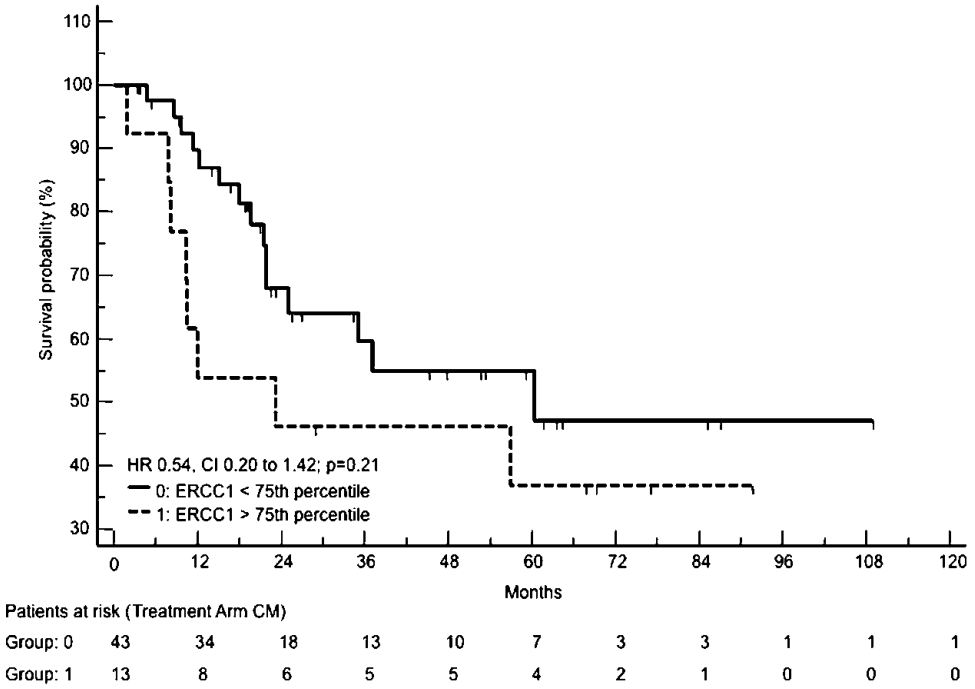

Figure 4. Kaplan-Meier plot estimating the progression-free survival of patients in the CM treatment arm. Differences in survival between the high- and the low-*ERCC1* expression group were analyzed with the log-rank test. The upper black line represents the low-expression group, whereas the lower broken line represents the high-expression group.

high risk of relapse despite radical cystoprostatectomy and systematic lymph node dissection. Chemotherapy has been proven efficacious in patients experiencing metastatic bladder cancer, with cisplatin being the most active agent. Accordingly, it was hypothesized that cisplatin-based chemotherapy applied before or after surgery for locally advanced bladder cancer would increase survival in this high-risk patient population. Although several randomized trials have been conducted, none of them conclusively demonstrated a significant survival benefit. However, improved progression-free survival was observed after adjuvant M-VAC compared with observation [29]. Relatively low patient numbers

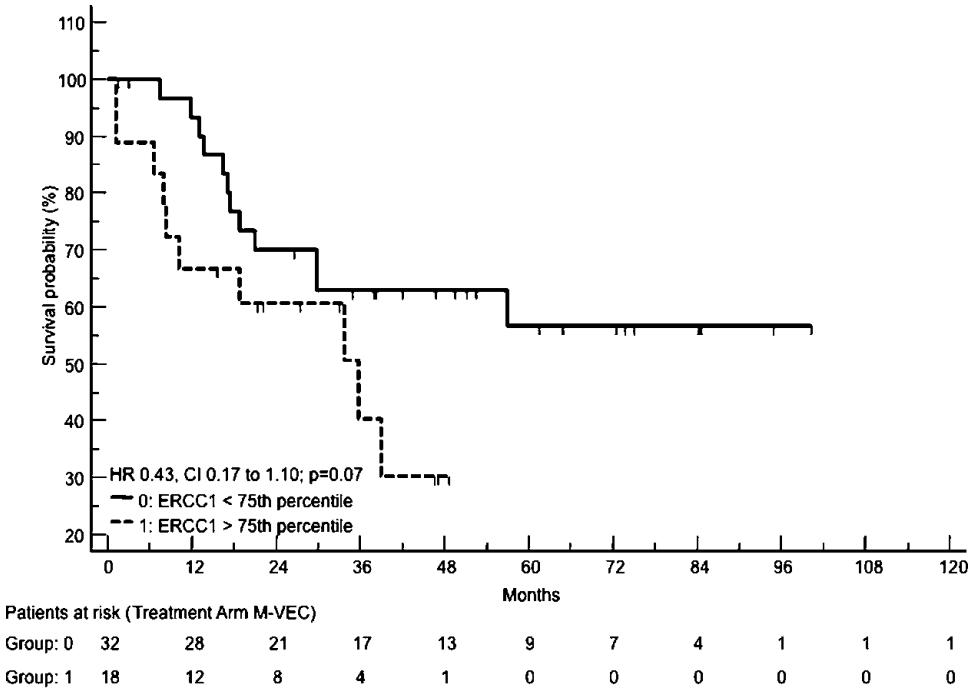

Figure 5. Kaplan-Meier plot estimating the progression-free survival of patients in the M-VEC treatment arm. Differences in survival between the high- and the low-*ERCC1* expression group were analyzed with the log-rank test. The upper black line represents the low-expression group, whereas the lower broken line represents the high-expression group.

Table 4. Cox Proportional Hazards Regression: Overall Survival, *ERCC1*.

Method	Stepwise
Enter variable if *P*	<.05
Remove variable if *P*	>.1
Sample size	107

Overall Model Fit

Null model −2 log likelihood	331.920
Full model −2 log likelihood	318.061
χ^2	13.859
df	2
Significance level	*P* = .0010

Coefficients and SE

Covariate	*b*	SE	*P*	Exp(*b*)	95% CI of Exp(*b*)
pN = 2	1.0649	0.3246	.001037	2.9006	1.5402-5.4627
Vascular invasion = 1	0.9975	0.4860	.04011	2.7115	1.0512-6.9945
Variables not included in the model					
ERCC1 > 75%					
pT = 1					
pT = 2					
pT = 4					
pN = 1					

Table 6. Cox Proportional Hazards Regression: Overall Survival, *MDR1* and *ERCC1*.

Method	Stepwise
Enter variable if *P*	<.05
Remove variable if *P*	>.1
Sample size	107

Overall Model Fit

Null model −2 log likelihood	331.920
Full model −2 log likelihood	308.682
χ^2	23.238
df	3
Significance level	*P* < .0001

Coefficients and SE

Covariate	*b*	SE	*P*	Exp(*b*)	95% CI of Exp(*b*)
pN = 2	1.0541	0.3267	.001254	2.8694	1.5174-5.4261
Vascular invasion	1.1277	0.4900	.02137	3.0886	1.1879-8.0306
MDR1 > 75%	1.0588	0.3294	.001306	2.8829	1.5167-5.4797
Variables not included in the model					
pT = 1					
pT = 2					
pT = 4					
pN = 1					
ERCC1 > 75%					

and brief follow-up in these studies could explain the lack of a measurable survival benefit from adjuvant chemotherapy. Alternatively, it is conceivable that only a subgroup of bladder cancer patients benefits from adjuvant chemotherapy, whereas it is of no effect or even detrimental for other patients. Thus, the development of biomarkers that are able to predict the presence or the absence of a benefit from adjuvant chemotherapy is of high importance for optimizing the care of patients with locally advanced bladder cancer. To this end, the present study was conducted to correlate *MDR1* and *ERCC1* gene expression with the outcome of patients undergoing adjuvant chemotherapy for muscle-invasive and/or nodal-metastasized urothelial bladder cancer within the randomized, prospective AUO-AB 05/95 phase 3 trial [6]. The *MDR1* and *ERCC1* genes were chosen for analysis because their encoded gene products have been implied as modifiers of the tumor cell response to the anticancer agents tested in AUO-AB 05/95.

The *MDR1* gene product Pgp is an energy-dependent efflux pump, which, among others, reduces intracellular concentrations of epirubicine and vinblastine, both of which were administered in the M-VEC arm of the trial. Moreover, methotrexate seems to be a substrate of Pgp when cells show deficient carrier-mediated methotrexate uptake [11]. Although cisplatin is not considered a *de novo* substrate of Pgp, some studies have suggested an altered expression of *MDR1* after cisplatin administration, possibly resulting in decreased cytotoxic

Table 5. Cox Proportional Hazards Regression: Progression-Free Survival, *ERCC1*.

Method	Stepwise
Enter variable if *P*	<.05
Remove variable if *P*	>.1
Sample size	105

Overall Model Fit

Null model −2 log likelihood	376.539
Full model −2 log likelihood	352.487
χ^2	24.052
df	3
Significance level	*P* < .0001

Coefficients and SE

Covariate	*b*	SE	*P*	Exp(*b*)	95% CI of Exp(*b*)
ERCC1 > 75%	0.8054	0.3082	.008969	2.2377	1.2268-4.0815
pN = 2	1.1446	0.3077	.0001991	3.1411	1.7240-5.7231
Vascular invasion	1.1676	0.4514	.009690	3.2142	1.3330-7.7507
Variables not included in the model					
pT = 1					
pT = 2					
pT = 4					
pN = 1					

Table 7. Cox Proportional Hazards Regression: Progression-Free Survival, *MDR1* and *ERCC1*.

Method	Stepwise
Enter variable if *P*	<.05
Remove variable if *P*	>.1
Sample size	105

Overall Model Fit

Null model −2 log likelihood	376.539
Full model −2 log likelihood	349.441
χ^2	27.097
df	3
Significance level	*P* < .0001

Coefficients and SE

Covariate	*b*	SE	*P*	Exp(*b*)	95% CI of Exp(*b*)
pN = 2	1.0742	0.3071	.0004684	2.9277	1.6087-5.3283
Vascular invasion	1.3289	0.4580	.003715	3.7769	1.5461-9.2264
MDR1 > 75%	1.0478	0.3234	.001194	2.8514	1.5178-5.3567
Variables not included in the model					
pT = 1					
pT = 2					
pT = 4					
pN = 1					
ERCC1 > 75%					

efficacy [30–33]. Whereas these studies might argue for a correlation between *MDR1* expression and resistance to platin compounds, additional reports failed to establish such an association [34]. Accordingly, the positive correlation between high *MDR1* expression and inferior survival and progression-free survival after adjuvant cisplatin-based chemotherapy as observed in our study does not automatically imply a causative role of Pgp. Moreover, it is tempting to speculate that the bulk of the prognostic or predictive value of *MDR1* expression is based on the inclusion of patients from the M-VEC arm. This hypothesis is supported by our findings studying the biomarkers separately in both treatment arms. *MDR1* expression performed significantly better as a discriminator of patient outcomes in the M-VEC arm than in the CM arm. In contrast, *ERCC1*, which encodes a gene product primarily modifying the cellular response to platin compounds and demonstrates significant association with progression-free survival in the whole study group, showed no difference between the two platin-based treatment arms. Because of the low patient numbers per group, these findings have to be interpreted with caution. However, they are in line with a potential biologic explanation for the association of *MDR1* expression and patient outcome after adjuvant chemotherapy.

As cisplatin is still regarded the main active drug in urothelial bladder cancer treatment, it is biologically plausible that the expression of an established modifier of the cellular platin response correlates with treatment efficacy. In our homogeneously defined and prospectively collected patient cohort, *ERCC1* expression was significantly and independently associated with progression-free survival, thus substantiating its role as biomarker for chemotherapy response in bladder cancer.

Our present study has been retrospectively conducted in samples collected from a completed clinical trial. Accordingly, the results may have been influenced by confounders that have occurred during the follow-up period but were not reported and by additional bias resulting from the fact that evaluable tissue blocks were only available from one third of the patients. Importantly, clinicopathologic parameters were equally balanced in the present study group and the entire trial population. Because of the lack of an observation arm in AUO-AB 05/95, it is impossible to decide whether expressions of *MDR1* and *ERCC1* are prognostic or predictive markers in this high-risk bladder cancer population. Interestingly, the results obtained with the two biomarkers applied in the present study can be corroborated by a biologic hypothesis, which is different from findings revealed by unselected expression analysis of thousands of parallel genes. This provides a strong rationale for implementing *MDR1* and *ERCC1* expression analysis in future trials of biomarker development in bladder cancer. To this end, the adjuvant setting is particularly suitable because tissue availability is not an issue. Because RT-PCR is a feasible method to retrieve results even from small tissue fractions, expressions of *MDR1* and *ERCC1* may also be used to better estimate which patients could benefit from neoadjuvant chemotherapy, even more because Hussain et al. [35] recently pointed out that administering chemotherapy to patients with resistant disease delays definitive local therapy while the disease progresses. In two large randomized trials, neoadjuvant chemotherapy with three courses of M-VAC before radical cystectomy provided a significant survival benefit [36–38]. However, this has not entered clinical practice in many centers, in part because of the substantial toxicities of the M-VAC regimen as well as the fear of tumor progression because of delayed surgery. As new drugs, such as gemcitabine and taxanes, have been introduced to the management of urothelial cancer, biomarkers in addition to *MDR1* and *ERCC1* may be required to provide a broader basis for the selection of treatment options for individualized patient care. This calls for further exploratory studies comparable to this one before embarking on a prospective biomarker trial. It will be of particular interest to our findings to explore the prognostic value of *MDR1* expression in a sufficiently powered bladder cancer population treated without anthracyclines and vinblastine. In conclusion, we have identified *MDR1* and *ERCC1* expressions as determined by real-time RT-PCR analysis as independent markers, which significantly correlate with overall survival and progression-free survival in patients undergoing cisplatin-based adjuvant chemotherapy after resection of locally advanced urothelial bladder cancer. This defines two promising and robust biomarkers to be prospectively validated toward the implementation of individualized care for bladder cancer patients.

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References

[1] Freiha F, Reese J, and Torti FM (1996). A randomized trial of radical cystectomy *versus* radical cystectomy plus cisplatin, vinblastine and methotrexate chemotherapy for muscle invasive bladder cancer. *J Urol* **155**, 495–499; discussion 499–500.

[2] Stockle M, Meyenburg W, Wellek S, Voges GE, Rossmann M, Gertenbach U, Thuroff JW, Huber C, and Hohenfellner R (1995). Adjuvant polychemotherapy of nonorgan-confined bladder cancer after radical cystectomy revisited: long-term results of a controlled prospective study and further clinical experience. *J Urol* **153**, 47–52.

[3] Skinner DG, Daniels JR, Russell CA, Lieskovsky G, Boyd SD, Nichols P, Kern W, Sakamoto J, Krailo M, and Groshen S (1991). The role of adjuvant chemotherapy following cystectomy for invasive bladder cancer: a prospective comparative trial. *J Urol* **145**, 459–464; discussion 464–467.

[4] Sternberg CN, Yagoda A, Scher HI, Watson RC, Ahmed T, Weiselberg LR, Geller N, Hollander PS, Herr HW, Sogani PC, et al. (1985). Preliminary results of M-VAC (methotrexate, vinblastine, doxorubicin and cisplatin) for transitional cell carcinoma of the urothelium. *J Urol* **133**, 403–407.

[5] Logothetis CJ, Dexeus FH, Finn L, Sella A, Amato RJ, Ayala AG, and Kilbourn RG (1990). A prospective randomized trial comparing MVAC and CISCA chemotherapy for patients with metastatic urothelial tumors. *J Clin Oncol* **8**, 1050–1055.

[6] Lehmann J, Retz M, Wiemers C, Beck J, Thuroff J, Weining C, Albers P, Frohneberg D, Becker T, Funke PJ, et al. (2005). Adjuvant cisplatin plus methotrexate *versus* methotrexate, vinblastine, epirubicin, and cisplatin in locally advanced bladder cancer: results of a randomized, multicenter, phase III trial (AUO-AB 05/95). *J Clin Oncol* **23**, 4963–4974.

[7] Metzger R, Leichman CG, Danenberg KD, Danenberg PV, Lenz HJ, Hayashi K, Groshen S, Salonga D, Cohen H, Laine L, et al. (1998). ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. *J Clin Oncol* **16**, 309–316.

[8] Bellmunt J, Paz-Ares L, Cuello M, Cecere FL, Albiol S, Guillem V, Gallardo E, Carles J, Mendez P, de la Cruz JJ, et al. (2007). Gene expression of *ERCC1* as a novel prognostic marker in advanced bladder cancer patients receiving cisplatin-based chemotherapy. *Ann Oncol* **18**, 522–528.

[9] Cobo M, Isla D, Massuti B, Montes A, Sanchez JM, Provencio M, Vinolas N, Paz-Ares L, Lopez-Vivanco G, Munoz MA, et al. (2007). Customizing cisplatin based on quantitative excision repair cross-complementing 1 mRNA expression: a phase III trial in non–small-cell lung cancer. *J Clin Oncol* **25**, 2747–2754.

[10] Simon G, Sharma A, Li X, Hazelton T, Walsh F, Williams C, Chiappori A, Haura E, Tanvetyanon T, Antonia S, et al. (2007). Feasibility and efficacy of molecular analysis–directed individualized therapy in advanced non–small-cell lung cancer. *J Clin Oncol* **25**, 2741–2746.

[11] de Graaf D, Sharma RC, Mechetner EB, Schimke RT, and Roninson IB (1996). P-glycoprotein confers methotrexate resistance in 3T6 cells with deficient carrier-mediated methotrexate uptake. *Proc Natl Acad Sci USA* **93**, 1238–1242.

[12] Pastan I, Willingham MC, and Gottesman M (1991). Molecular manipulations of the multidrug transporter: a new role for transgenic mice. *FASEB J* **5**, 2523–2528.
[13] Walter RB, Gooley TA, van der Velden VH, Loken MR, van Dongen JJ, Flowers DA, Bernstein ID, and Appelbaum FR (2007). CD33 expression and P-glycoprotein–mediated drug efflux inversely correlate and predict clinical outcome in patients with acute myeloid leukemia treated with gemtuzumab ozogamicin monotherapy. *Blood* **109**, 4168–4170.
[14] Walter RB, Raden BW, Hong TC, Flowers DA, Bernstein ID, and Linenberger ML (2003). Multidrug resistance protein attenuates gemtuzumab ozogamicin–induced cytotoxicity in acute myeloid leukemia cells. *Blood* **102**, 1466–1473.
[15] Pallis M and Russell N (2000). P-glycoprotein plays a drug-efflux–independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. *Blood* **95**, 2897–2904.
[16] Ashariati A (2008). Polymorphism C3435T of the *MDR-1* gene predict response to preoperative chemotherapy in locally advanced breast cancer with Her2/*neu* expression. *Acta Med Indones* **40**, 187–191.
[17] Cizmarikova M, Wagnerova M, Schonova L, Habalova V, Kohut A, Linkova A, Sarissky M, Mojzis J, Mirossay L, and Mirossay A (2010). *MDR1* (C3435T) polymorphism: relation to the risk of breast cancer and therapeutic outcome. *Pharmacogenomics J* **10**(1), 62–69.
[18] Abolhoda A, Wilson AE, Ross H, Danenberg PV, Burt M, and Scotto KW (1999). Rapid activation of *MDR1* gene expression in human metastatic sarcoma after *in vivo* exposure to doxorubicin. *Clin Cancer Res* **5**, 3352–3356.
[19] Baldini N, Scotlandi K, Barbanti-Brodano G, Manara MC, Maurici D, Bacci G, Bertoni F, Picci P, Sottili S, Campanacci M, et al. (1995). Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. *N Engl J Med* **333**, 1380–1385.
[20] Goebell PJ (2009). Outcomes and response to therapy in bladder cancer. Are biomarkers of any help? *Minerva Urol Nefrol* **61**, 91–107.
[21] Petrylak DP, Scher HI, Reuter V, O'Brien JP, and Cordon-Cardo C (1994). P-glycoprotein expression in primary and metastatic transitional cell carcinoma of the bladder. *Ann Oncol* **5**, 835–840.
[22] Sobin LH and Fleming ID (1997). TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* **80**, 1803–1804.
[23] Hoffmann AC, Danenberg KD, Taubert H, Danenberg PV, and Wuerl P (2009). A three-gene signature for outcome in soft tissue sarcoma. *Clin Cancer Res* **15**, 5191–5198.
[24] Somlo G, Chu P, Frankel P, Ye W, Groshen S, Doroshow JH, Danenberg K, and Danenberg P (2008). Molecular profiling including epidermal growth factor receptor and p21 expression in high-risk breast cancer patients as indicators of outcome. *Ann Oncol* **19**, 1853–1859.
[25] Hoffmann AC, Mori R, Vallbohmer D, Brabender J, Klein E, Drebber U, Baldus SE, Cooc J, Azuma M, Metzger R, et al. (2008). High expression of HIF1a is a predictor of clinical outcome in patients with pancreatic ductal adenocarcinomas and correlated to PDGFA, VEGF, and bFGF. *Neoplasia* **10**, 674–679.
[26] Metz CE (1978). Basic principles of ROC analysis. *Semin Nucl Med* **8**, 283–298.
[27] Zweig MH and Campbell G (1993). Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* **39**, 561–577.
[28] Angulo JC, Lopez JI, Grignon DJ, and Sanchez-Chapado M (1995). Muscularis mucosa differentiates two populations with different prognosis in stage T1 bladder cancer. *Urology* **45**, 47–53.
[29] Lehmann J, Franzaring L, Thuroff J, Wellek S, and Stockle M (2006). Complete long-term survival data from a trial of adjuvant chemotherapy *vs* control after radical cystectomy for locally advanced bladder cancer. *BJU Int* **97**, 42–47.
[30] Demeule M, Brossard M, and Beliveau R (1999). Cisplatin induces renal expression of P-glycoprotein and canalicular multispecific organic anion transporter. *Am J Physiol* **277**, F832–F840.
[31] Takara K, Tsujimoto M, Kokufu M, Ohnishi N, and Yokoyama T (2003). Up-regulation of *MDR1* function and expression by cisplatin in LLC-PK1 cells. *Biol Pharm Bull* **26**, 205–209.
[32] van den Broek GB, Wildeman M, Rasch CR, Armstrong N, Schuuring E, Begg AC, Looijenga LH, Scheper R, van der Wal JE, Menkema L, et al. (2009). Molecular markers predict outcome in squamous cell carcinoma of the head and neck after concomitant cisplatin-based chemoradiation. *Int J Cancer* **124**, 2643–2650.
[33] Pu YS, Tsai TC, Cheng AL, Tsai CY, Tseng NF, Su IJ, Hsieh CY, and Lai MK (1996). Expression of *MDR-1* gene in transitional cell carcinoma and its correlation with chemotherapy response. *J Urol* **156**, 271–275.
[34] Ren L, Xiao L, and Hu J (2007). *MDR1* and *MDR3* genes and drug resistance to cisplatin of ovarian cancer cells. *J Huazhong Univ Sci Technolog Med Sci* **27**, 721–724.
[35] Hussain MH, Wood DP, Bajorin DF, Bochner BH, Dreicer R, Lamm DL, O'Donnell MA, Siefker-Radtke AO, Theodorescu D, and Dinney CP (2009). Bladder cancer: narrowing the gap between evidence and practice. *J Clin Oncol* **27**, 5680–5684.
[36] Grossman HB, Natale RB, Tangen CM, Speights VO, Vogelzang NJ, Trump DL, deVere White RW, Sarosdy MF, Wood DP Jr, Raghavan D, et al. (2003). Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. *N Engl J Med* **349**, 859–866.
[37] International Collaboration of Trialists (1999). Neoadjuvant cisplatin, methotrexate, and vinblastine chemotherapy for muscle-invasive bladder cancer: a randomised controlled trial. *Lancet* **354**, 533–540.
[38] Wallace DM, Raghavan D, Kelly KA, Sandeman TF, Conn IG, Teriana N, Dunn J, Boulas J, and Latief T (1991). Neo-adjuvant (pre-emptive) cisplatin therapy in invasive transitional cell carcinoma of the bladder. *Br J Urol* **67**, 608–615.